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## PURIFICATION AND CHARACTERIZATION OF THE TYPE III SECRETION SYSTEM PROTEIN FROM *BURKHOLDERIA MALLEI*

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# PURIFICATION AND CHARACTERIZATION OF THE TYPE III SECRETION SYSTEM PROTEIN FROM *BURKHOLDERIA MALLEI*

## 1. INTRODUCTION

*Burkholderia mallei*, which is a Gram-negative pathogen that infects horses and humans, is the causative agent of glanders.<sup>1</sup> This bacterium is classified as a select agent by the Centers for Disease Control and Prevention (CDC; Atlanta, GA) because of its high infectivity rates and past use as a biological weapon.<sup>2</sup> To deliver virulence into host tissues, the pathogen uses two major virulence systems, the type III secretion system (T3SS) and the type VI secretion system (T6SS).<sup>3,4</sup> The T3SS is found in many Gram-negative bacteria including *Yersinia*, *Salmonella*, *Shigella*, and enteropathogenic *Escherichia coli*.<sup>5-9</sup> All pathogens that contain T3SS are of interest to the U.S. Army, and research into preventive and therapeutic measures against T3SS-containing pathogens are being actively pursued. The mechanism by which the T3SS recognizes and transports virulence factors extracellularly is not known, but it depends on the presence of an active ATPase (enzyme that catalyzes the decomposition of adenosine triphosphate [ATP]) contained in the BsaS subunit.<sup>10-11</sup> The energy for the transport is thought to be derived from proton gradients, but some controversy remains. Based on similarities between the T3SS proteins that have been identified to date and the well-known flagellar ATPase system, we have hypothesized that the T3SS ATPase forms hexameric assemblies as catalytic units.<sup>12-15</sup> Although the flagellar ATPase can transition to a monomer, the hexameric form is not a necessary requirement for catalysis. A possible explanation is that the hexameric complex is required to uncouple the effectors from the chaperone-effector complex, and the ATPase is contained in a single unit. On the basis of the *in silico* modeling results, conducted on homologous *Yersinia pestis* T3SS YscN ATPase, blocking full closure on the C-terminal of the protein or introduction of steric hindrances from adjacent subunits will render the oligomer either unable to bind or hydrolyze adenosine triphosphate (ATP). This prediction will be confirmed by introducing small changes in the nucleotide binding site and the adjacent subunit through site-directed mutagenesis of cloned *B. mallei* BsaS. If the prediction is confirmed, it would help to explain how ATP hydrolysis may be coupled to molecular movements.

The T3SS in *B. mallei* is encoded chromosomally, and the key components have been identified by analysis of genetic deletions in the T3SS operon.<sup>3</sup> Models predict that the T3SS is an Mg<sup>2+</sup>-dependent ATPase. We propose to examine the validity of the models by expressing BsaS in *E. coli*, measuring its activity *in vitro*, and correlating it with conformational changes. If this is successful, a potential screening approach that targets the T3SS ATPase may then be available to identify treatments for glanders. In the second year of the proposed project, in conjunction with our collaborators at the New York Structural Biology Center, we intend to crystallize and identify the structure of the native and mutationally inactive T3SS BsaS ATPase.

## 2. METHODS AND MATERIALS

### 2.1 Gene Cloning

The catalytic domain and the full-length, open-reading frame fragments of the *bsaS* gene from the *B. mallei* ATCC 233344 strain were optimized for codon usage and *E. coli* expression using in silico methods. The gene fragments were synthesized chemically, cloned into a pMAL (New England BioLabs, Inc. [NEBioLabs]; Ipswich, MA) vector, and verified by sequencing (GenScript USA, Inc.; Piscataway, NJ). The final construct included a 10× histidine (10× His) affinity tag at the carboxyl (C)-terminus and a cleavable (enterokinase) maltose-binding protein (MBP) fusion at the amino (N)-terminus.

### 2.2 Protein Expression

The construct was transformed into *E. coli* T7 Express cells (NEBioLabs), and the protein expression was performed using the terrific broth (TB) (Novagen, a product of EMD Millipore; Billerica, MA) auto-induction medium supplemented with 50 mg/mL ampicillin at 22 °C. After overnight growth, the cells were harvested by centrifugation and stored at –80 °C. To purify the full length protein, the cells were thawed and resuspended in a buffer consisting of 2× trisaminomethane (Tris)-buffered saline (TBS: 10 mM Tris, pH 8.0, and 150 mM of NaCl), 40 mM imidazole, and 10% glycerol. This solution was disrupted in a Microfluidizer (Microfluidics; Newton, MA), and the debris was removed by centrifugation at 28,000× *g*, 4 °C for 2 h. The lysate was filtered through a 0.45 mm sterile filter and applied on a HisTrap crude FF column (GE Healthcare Biosciences; Pittsburgh, PA) that was connected to an ÄKTAExpress protein-purification system (GE Healthcare). The protein was eluted from the HisTrap crude FF column with a step gradient of 500 mM imidazole and loaded directly on a HiLoad Superdex 200 26/60 column (GE Healthcare) equilibrated in 2× TBS and 10% glycerol. The peaks containing the protein were pooled and applied to an MBPTrap high-performance (HP) column (GE Healthcare) and eluted with a linear gradient of 10 mM maltose. The fractions containing the protein were pooled, the buffer was exchanged into 2× TBS and 10% glycerol, and then this solution was concentrated using Amicon Ultra 15 (10 kDa cutoff) concentrators (EMD Millipore). Purity was verified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentrated protein was aliquoted and stored at –20 °C. The catalytic domain of BsaS was purified in an analogous way but, because of the higher affinity for the amylose resin (MBPTrap HP column), the procedure was performed in an automated mode using the following four steps: eluting through a HisTrap crude FF column, desalting, applying to an MBPTrap HP column, and then applying to a HiLoad Superdex 200 26/60 column.

### 2.3 Enzymatic Activity

Enzymatic activity in the cells was measured using an EnzCheck Protease Assay kit (Invitrogen, now Life Technologies; Grand Island, NY). This procedure was performed after phosphate was released from ATP using a coupled assay that relied on incorporating the phosphate into a chromogenic substrate. Typically, the reaction was performed at 37 °C in 1× TBS buffer in the presence of 2 mM Mg<sup>2+</sup>. At specific time points, aliquots were withdrawn,

quenched with 2 mM ethylenediaminetetraacetic acid (EDTA), and stored at  $-20^{\circ}\text{C}$  for further processing.

### 3. RESULTS

The catalytic domain of the BsaS protein was purified to homogeneity (Figures 1 and 2). The ATPase activity of the protein (Figure 3) was found to be nonlinear when plotted against the enzyme concentration (Figure 4), and a simple kinetic model could not be applied to calculate the apparent Michaelis constant ( $K_m$ ) and maximum rate achieved by the system ( $V_{\max}$ ). The best approximation was obtained when the Lineweaver-Burk plot was fitted to each linear portion of the activity plot (Figure 5).



Figure 1. The last step of BsaS protein purification. The pooled fractions from size-exclusion chromatography were applied on an MBPTrap HP (GE Healthcare) affinity column and eluted with a linear gradient of 10 mM of maltose.

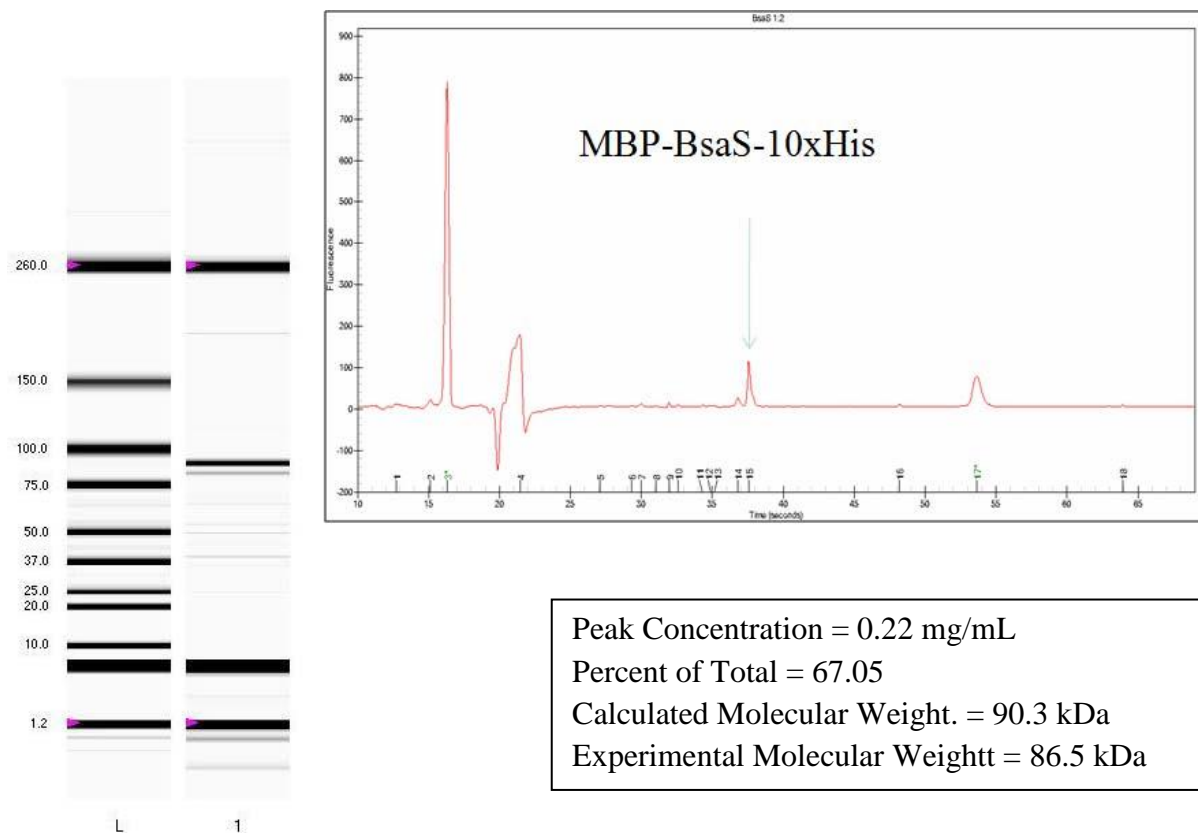


Figure 2. SDS-PAGE results for purified BsaS protein. The gel was run on an Experion (Bio-Rad Laboratories; Hercules, CA) automated electrophoresis system.

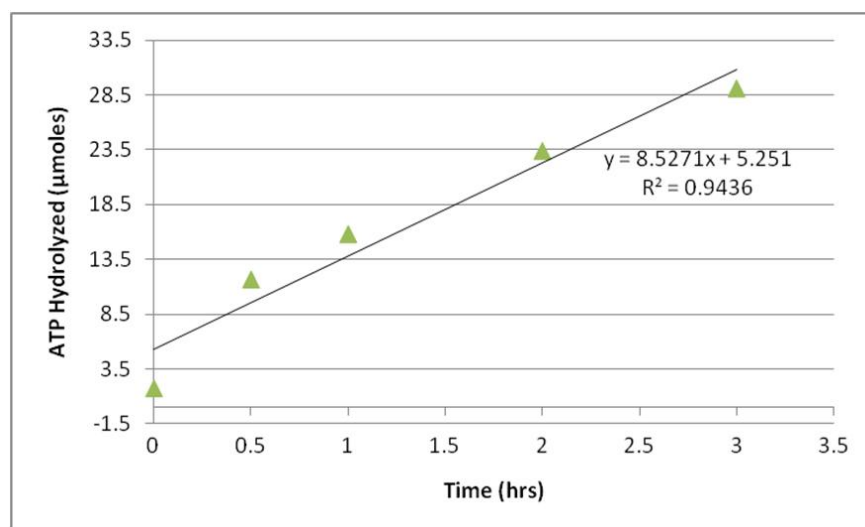


Figure 3. A representative kinetic trace showing ATP hydrolysis of the BsaS protein. The hydrolysis was quantified by following the release of phosphate in a coupled reaction of BsaS protein in 1× TBS and 2 mM of  $Mg^{2+}$  at 37 °C. Total amount of BsaS protein used was 6.3 μg.

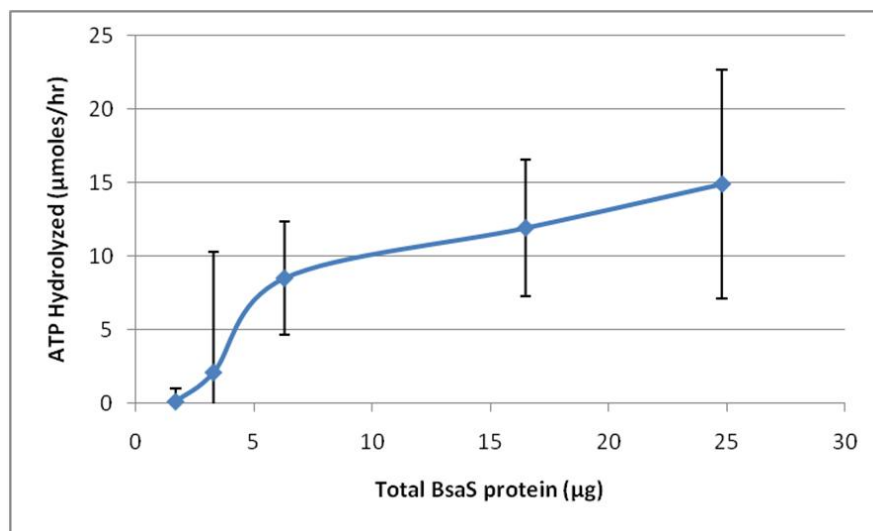


Figure 4. Nonlinear plot of initial ATP hydrolysis versus protein concentration. The hydrolysis was quantified by following the release of phosphate in a coupled reaction, performed in 1× TBS and 2 mM of  $Mg^{2+}$  at 37 °C.

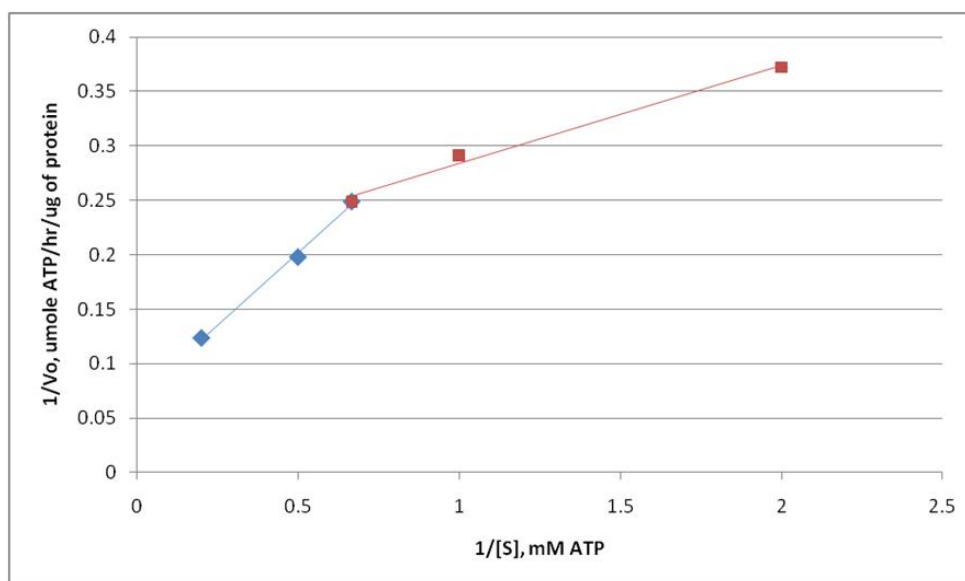


Figure 5. A plot showing allostery of the ATP hydrolysis by BsaS. The apparent  $K_m$  and  $V_{max}$  values were determined from Lineweaver-Burk plots for separate ranges of substrate concentrations. The hydrolysis was quantified by following the release of phosphate in a coupled reaction of BsaS protein in 1× TBS and 2 mM of  $Mg^{2+}$  at 37 °C. Range of ATP concentration was 0.2–5 mM. Total concentration of BsaS protein was 6.3 μg.

#### 4. DISCUSSION

Initial attempts to express the protein fragments using native gene fragments were not successful due to the extremely low protein production yielded by the *E. coli* (R. Dorsey, unpublished results). Therefore, the gene was optimized for *E. coli* expression, and all cystine residues were replaced with serine to minimize protein aggregation. Model building of the BsaS protein suggested that none of the cystine residues formed intramolecular disulfide bridges (R. Dorsey, unpublished data). In addition, reconstruction of the putative hexameric oligomer, based on the rigid-body protein docking and on the *Salmonella* FliI putative hexamer, did not lead to the suggestion that the cystine residues would be involved in intermolecular disulfide bridge formation (W. Swietnicki, unpublished data). Therefore, the replacements were judged to be justified.

The expressed BsaS protein was shown to have ATPase activity, which was in agreement with the computational predictions. In orthologous T3SSs, the ATPases are thought to be responsible for the release of virulence factors from their complexes, with specific chaperones before transport through the translocation pore from the bacterial cytosol and directly into the mammalian cells. The N-terminal part on the ATPases is most likely involved in the release process, but the details are not known. Based on the homology modeling and available structural data, it was determined that the catalytic and N-terminal domains of the ATPases are apparently separated by a flexible linker. The release of the chaperones may require an oligomerization of the ATPase because oligomeric states are observed in all homologous ATPases that have been purified to date and because the native conformation of plant-like T3SS HrcN ATPase is a dodecamer. The full-length T3SS ATPases also show catalytic activity that is associated with the hexameric form of the protein. The nonlinear dependence of activity on the enzyme concentration, which was also observed for the T3SS ATPases and in our experiments, may reflect the dependence of the catalytic activity on the tertiary arrangements of the complex. The tertiary arrangement may be influenced by the N-terminal domain of the protein and may involve conformational arrangements within the complex. The details of the mechanism are currently under investigation.

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## ACRONYMS AND ABBREVIATIONS

10× His	10× histidine affinity tag
ATP	adenosine triphosphate
ATPase	enzyme that catalyzes the decomposition of adenosine triphosphate
C-terminus	carboxyl-terminus
CDC	Centers for Disease Control and Prevention
EDTA	ethylenediaminetetraacetic acid
HP	high performance
$K_m$	Michaelis constant (used in enzyme kinetics)
MBP	maltose-binding protein
NEBioLabs	New England BioLabs, Inc.; Ipswich, MA
N-terminus	amino-terminus
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T3SS	type III secretion system (pathogen virulence)
T6SS	type VI secretion system (pathogen virulence)
TB	terrific broth (medium)
TBS	Tris-buffered saline
Tris	trisaminomethane
$V_{max}$	maximum rate achieved by the system (used in enzyme kinetics)





